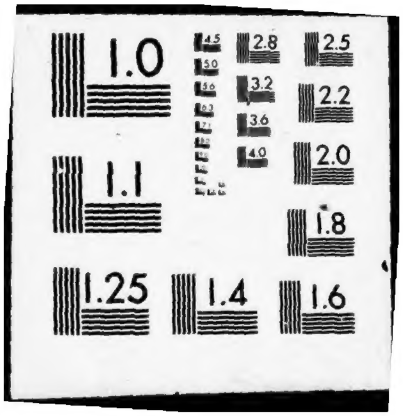
AD-A034 199 CALIFORNIA UNIV LOS ANGELES DEPT OF MICROBIOLOGY AND--ETC
SYPHILIS VACCINE AND IMMUNE MECHANISMS.(U)
DEC 76 J N MILLER N00014-76-C-0148
NL

UNCLASSIFIED

1 OF 1
AD-A
034 199

END
DATE
FILMED
2-26-77
NTIS


1.0
4.5
5.0
5.6
6.3
2.8
3.2
3.6
4.0
2.5
2.2
2.0
1.1
1.8
1.25
1.4
1.6

U.S. DEPARTMENT OF COMMERCE
National Technical Information Service

AD-A034 199

SYPHILIS VACCINE AND IMMUNE MECHANISMS

CALIFORNIA UNIVERSITY, LOS ANGELES

31 DECEMBER 1976

012003

ADA034199

OFFICE OF NAVAL RESEARCH

Contract N00014-76-C-0148
Task No. 136-912

ANNUAL REPORT NO. 5

by

James N. Miller, Ph.D.

UNIVERSITY OF CALIFORNIA AT LOS ANGELES
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
LOS ANGELES, CALIFORNIA 90024

JANUARY 1, 1976 - DECEMBER 31, 1976

DDC
RECEIVED
JAN 11 1977
A

REPRODUCTIONS IN WHOLE OR IN PART IS PERMITTED
FOR ANY PURPOSE OF THE UNITED STATES GOVERNMENT

THIS DOCUMENT HAS BEEN APPROVED FOR PUBLIC
RELEASE: ITS DISTRIBUTION IS UNLIMITED

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U. S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

1

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER Annual Report No. 5	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) SYPHILIS VACCINE AND IMMUNE MECHANISMS		5. TYPE OF REPORT & PERIOD COVERED Annual Report No. 5 1/1/76 - 12/31/76
		6. PERFORMING ORG. REPORT NUMBER Annual Report No. 5
7. AUTHOR(s) James N. Miller, Ph.D.		8. CONTRACT OR GRANT NUMBER(s) N00014-76-C-0148
9. PERFORMING ORGANIZATION NAME AND ADDRESS James N. Miller, Ph.D., UCLA School of Medicine University of California at Los Angeles Los Angeles, California 90024		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS NR 136-912
11. CONTROLLING OFFICE NAME AND ADDRESS Office of Naval Research 800 No. Quincy Street Arlington, Virginia 22217		12. REPORT DATE 1 Jan. 1976 - 31 Dec. 1976
		13. NUMBER OF PAGES 85
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Distribution of this document is unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

Same

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

See Attached Reversed Side

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

See Attached Reversed Side

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE
S/N 0102-014-6601

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

19. KEY WORDS

Immunity - Experimental and Human Syphilis.

- A. Vaccine
  - 1. γ-irradiated *Treponema pallidum*, liquid nitrogen preservation
  - 2. Adjuvant
  - 3. Homologous Acquired Resistance
  - 4. Antibody Response
- B. Mechanisms of Immunity
  - 1. Humoral
    - a. Passive Protection
    - b. "In Vitro"-"In Vivo" Neutralization
  - 2. Cell-Mediated
    - a. Direct Cytotoxicity
    - b. Anti-Rabbit Thymocyte Globulin
- C. Purification - *Treponema pallidum*
- D. "In Vitro" Cultivation in Tissue Culture Monolayers

20. ABSTRACT

I. DEVELOPMENT OF AN EFFECTIVE AND PRACTICAL VACCINE AGAINST EXPERIMENTAL AND HUMAN SYPHILIS

A. No immunity to the intradermal challenge of 542 *T. pallidum* was demonstrated among rabbits vaccinated intramuscularly at 4-week intervals with a total of 4 vaccine doses, each containing an equal volume of 4.3 to 5 X 10^9 γ-irradiated (650,000 rads), liquid nitrogen-preserved *T. pallidum* and an alginate-gluconate adjuvant; each of the vaccinated animals developed VDRL antibody.

B. The possibility that soluble surface immunogen(s) detach from the γ-irradiated treponemes during processing prior to storage has led to the design of experiments utilizing supernatants, obtained during preparation of vaccine, as immunizing agents.

II. MECHANISM(S) OF THE IMMUNE RESPONSE IN EXPERIMENTAL AND HUMAN SYPHILIS

A. Humoral Mechanism(s)

1. Experimental Syphilis

a. Humoral mechanism(s) operative during the immune process have been demonstrated by the use of both passive protection and "in vitro"-"in vivo" neutralization techniques. (See Annual Reports Nos. 3 and 4.)

b. As a prelude to further immune mechanism studies, it has been shown that rabbits injected with graded doses of T. pallidum (10^6, 10^5, 10^4, and 10^3) develop lesions essentially within the same incubation periods as rabbits given single doses, suggesting that the incubation period required for lesion development is independent of the influence from other sites as long as the inoculum contains at least 10^3 treponemes; however, the later course of lesion development was shown to be influenced by other lesions. In further graded dose studies, it was found that lesions which develop from 10^6 inocula do, indeed, influence the development of lesions from inocula containing 10^3, 10^2 and 10 treponemes. These studies are continuing.

2. Human Syphilis

a. Although a technique has been developed for determining the relationship of neutralizing antibody to immunity in patients, it involves the use of 30% unheated human serum due to the non-specific treponemicidal activity operative when a 90% concentration is employed. (See Annual Report No. 4.)

b. Preliminary studies have shown that absorption of unheated normal human serum with non-pathogenic *Treponema phagedenis var. reiteri* removes the non-specific heat-stable treponemicidal factor(s). Inasmuch as absorption in this manner might allow the use of maximal concentrations of unheated sera in the neutralization assay, these studies are continuing.

B. Cell-Mediated Mechanism(s)

1. Human Syphilis

a. Preliminary experiments have shown that peripheral lymphocytes from patients with latent syphilis may have a significant cytotoxic effect upon T. pallidum. (See Annual Reports Nos. 3 and 4.)

b. Studies to determine whether cytotoxicity can be correlated with the human immune process are being planned.

2. Experimental Syphilis

a. In experiments designed to determine whether a treponemicidal effect of immune lymphocytes is mediated by specific treponemal antibody, it was found that the use of immune serum dilutions in combination with both normal and immune lymphocytes does not result in the inactivation of T. pallidum.

b. Studies are underway to confirm and extend the finding that immune lymphocytes "primed" for 24 hours by γ-irradiated T. pallidum exerts a specific cytotoxic effect upon virulent T. pallidum. (See Annual Report No. 4.)

c. No significant differences were observed in the development of lesions among rabbits inoculated intravenously and daily with goat anti-rabbit thymocyte globulin (ATG) as compared to those inoculated with normal goat globulin or saline. (See Annual Report No. 4.)

d. Studies have been planned to obtain a highly specific ATG by absorption techniques as a prelude to further experiments to determine whether susceptibility can be enhanced in this manner.

Treponema

cont p. 4

III. PURIFICATION AND "IN VITRO" TISSUE CULTIVATION OF "T. PALLIDUM", NICHOLS STRAIN

A. Purification by Discontinuous Ficoll Density Gradients

As a prelude to the design of a more effective Ficoll gradient for purification of *T. pallidum*, the equilibrium buoyant density of the organism was determined to be 1.190 to 1.193 gms/ml utilizing linear sucrose gradients. (See Annual Report No. 4.)

B. "In Vitro" Cultivation in Tissue Culture Monolayers

1. It has been shown that *T. pallidum* forms a strong attachment to monolayer cell surfaces. Further, the treponemes appear to show a strikingly selective preference for epithelioid rather than fibroblastic cells in primary monolayer cultures derived from normal rabbit testis; this may be a significant requirement for *T. pallidum* multiplication.

2. Transmission electron microscope studies have demonstrated for the first time, the active penetration of *T. pallidum* into primary monolayer cells derived from normal rabbit testis.

3. As a prelude to cell monolayer-treponeme interaction experiments, it has been shown that MEM containing 50% fresh, heat-inactivated normal rabbit serum and 1.0 mM dithiothreitol, incubated at 34°C in an atmosphere of 2.88% O_2, 5% CO_2 and 92.12% N_2, is presently the most optimally defined tissue culture medium and environment for best maintenance of motility and virulence. Further, the medium does not damage cell cultures.

This report contains a significant bibliography.

ANNUAL REPORT NO. 5

BACKGROUND AND RESEARCH ACCOMPLISHMENTS

I. DEVELOPMENT OF AN EFFECTIVE AND PRACTICAL VACCINE AGAINST EXPERIMENTAL AND HYMAN SYPHILIS (Objective No. 1)

A. Immunization of Rabbits with Liquid-Nitrogen Preserved, γ-Irradiated "Treponema pallidum", Nichols Strain, in a Reduced Injection Schedule (with and without Adjuvants).

1. Degree of Homologous Acquired Resistance

The basis for continued studies toward the development of a practical vaccine utilizing γ-irradiated T. pallidum as the immunogen has now been firmly established (Miller, 1973; Miller, 1976). The publications and Renewal Application No. 3 (6/24/74) have detailed (a) the complete immunity to homologous challenge accomplished by immunizing rabbits intravenously over a 37-week period with 60 injections containing a total of 3.71×10^9 freshly isolated T. pallidum, Nichols strain, γ-irradiated with 650,000 rads, (b) the necessity for reducing the number of immunizing injections with or without adjuvants, (c) the rationale and successfully developed procedures for preservations of large numbers of irradiated treponemes for relatively long periods of time in liquid nitrogen, (d) the development of some degree of homologous acquired resistance following intravenous and intramuscular vaccination with liquid nitrogen-preserved 650,000 rad γ-irradiated T. pallidum, Nichols strain, employing a practical time schedule, (e) the enhancing effect of a sodium alginate-calcium gluconate adjuvant upon the immune response to homologous challenge following intramuscular vaccination, and (f) the presence of relatively few infectious treponemes within the preserved, irradiated treponemal vaccine, suggesting the occurrence of DNA repair during liquid nitrogen storage.

Table I summarizes the comparative data supporting the conclusion that (a) partial immunity developed among 11 animals receiving non-infectious, γ-irradiated (650,000 rads), liquid

TABLE I

Results of Challenge Inoculation in Rabbits Immunized With ~ 12 X 10^9 Liquid Nitrogen-Preserved, γ-Irradiated *T. pallidum*, With and Without Adjuvant, in Two Separate Experiments[a]

Days After Challenge	Development of Lesions					
	With Adjuvant[b]			Without Adjuvant		
	Accumulative Number of Positive Sites[c]					
	Intramuscular (Non-Infectious)	Intramuscular (Infectious)	Controls	Intravenous	Intramuscular	Controls
	11 Rabbits[d]	1 Rabbit[e]	12 Rabbits	9 Rabbits[f]	8 Rabbits[g]	12 Rabbits
7						3/48 (6%)
8						4/48 (8%)
9						5/48 (10%)
10			17/48 (35%)			14/48 (29%)
11			30/48 (63%)			24/48 (50%)
12			40/48 (83%)			32/48 (67%)
13			43/48 (90%)			46/48 (96%)
14	6/44 (14%)		48/48 (100%)			48/48 (100%)
15	8/44 (18%)					
16	14/44 (32%)				5/32 (16%)	
17	19/44 (43%)			3/36 (8%)	13/32 (41%)	
18	23/44 (52%)	1/4 (25%)		14/36 ([illegible])	32/32 (100%)	
19	26/44 (59%)	3/4 (75%)		20/36 ([illegible])		
20	34/44 (77%)	4/4 (100%)		32/36 ([illegible])		
21	38/44 (86%)					
22	40/44 (91%)					
23	42/44 (95%)					
24	42/44 (95%)					
25	44/44 (100%)					
Mean Time (Days)	18.3	19.5	11.3	18.8	17.4	11.2

---

[a] γ-Irradiation dosage, 630,000 rads; challenged by the intradermal route at 4 sites.

Challenge dose per site: "Without Adjuvant" Experiment - 1500 *T. pallidum*
"With Adjuvant" Experiment - 2340 *T. pallidum*

[b] Vaccine Combined with equal volume of 4% sodium alginate - 0.67% calcium alginate adjuvant containing 0.4% phenol as preservative.

[c] Numerator = Sites Positive; Denominator = Total Sites.

[d] Eight rabbits developed flat, erythematous, atypical lesions at all sites.

[e] Received vaccine containing infectious treponemes; developed flat, erythematous, atypical lesions at all sites following challenge.

[f] One rabbit failed to develop lesions at any site during the 88-day observation period.

[g] One rabbit developed flat, erythematous, atypical lesions at all sites.

nitrogen-preserved T. pallidum, Nichols strain, in an alginate-gluconate adjuvant, and (b) the adjuvant exerted an enhancing effect upon the immune response to homologous challenge when compared to the response of rabbits immunized intramuscularly with the same total dosage of vaccine, but without adjuvant. Evidence of partial immunity among the 11 animals receiving non-infectious vaccine was demonstrated by a significant delay in the development of lesions, ranging from 3 (2.7) to 14 (13.7) days in each of the immunized rabbits compared to the average time of lesion development in the non-immunized controls. Further, 8 of the 11 vaccinated rabbits developed relatively flat, erythematous, atypical lesions which healed at a time when 10 of the 12 control rabbits were exhibiting ulcerative lesions. It is interesting to note that the animals immunized with vaccine containing infectious treponemes did not develop a greater immunity than those animals which were partially protected by means of completely inactivated organisms; atypical lesions developed after a delay in the incubation period of 7 (7.2) days compared to the control animals and persisted longer than the atypical lesions which occurred in the other "test" animals.

The enhancing effect was suggested by the findings that (a) none of the 8 rabbits vaccinated intramuscularly without adjuvants exhibited a delay of more than 7 (6.8) days in the appearance of lesions compared to the average time of lesions development in the non-immunized controls, and (b) 8 of the 11 animals vaccinated with the treponeme-adjuvant mixture developed flat, erythematous, atypical lesions in contrast to their development in only 1 of the 8 animals vaccinated intramuscularly without adjuvants.

These results pointed to the necessity for further experiments employing modifications in the spacing and numbers of vaccine-adjuvant injections as well as the use of smaller challenge inocula in order to determine whether a clearly demonstrable complete immunity can be achieved. The finding of infectious treponemes in the preserved vaccine also indicated the necessity for (a) determining the extent of infectiousness within the remaining stored vaccine doses irradiated at this level and (b) exposing the treponemes to a total γ-irradiation dosage greater than 650,000 rads prior to processing and storage in liquid nitrogen for further immunization experiments.

In an effort to determine the extent of infectiousness within the preserved vaccine, 24 vials (1 ml per vial) of T. pallidum γ-irradiated with 650,000 rads and preserved in liquid nitrogen for 7-10 months, were thawed and pooled to give a concentration of 3×10^9 treponemes per ml. The morphology of the treponemes was excellent and the motility equivalent to that of the preserved treponemal vaccine used in the previously described adjuvant experiment. Each of 10 serologically non-reactive New Zealand white male rabbits was inoculated intratesticularly with 1 ml of the vaccine per testis or a total of 6×10^9 treponemes. None of the 9 surviving rabbits showed evidence of infection during the 3 months of observation as measured by negative darkfield examination and TPI tests (1 animal died from unknown causes following 2 months of observation during which darkfield and TPI results were also negative). At the end of the 3-month observation period, the popliteal lymph nodes and testes of the 9 animals were removed and inoculated intratesticularly into serologically non-reactive, normal rabbits. None of the recipient animals showed evidence of infection during the 6-month observation period as measured by negative darkfield examination and TPI tests.

Thus, the available evidence indicated that there were relatively few infectious treponemes present in the 650,000 rad γ-irradiated vaccine, and suggested that exposure to a total γ-irradiation dosage of 1,000,000 rads prior to processing and storage in liquid nitrogen might effect complete inactivation of the organisms without the possibility of DNA repair during storage.

Freshly isolated *T. pallidum*, Nichols strain, were γ-irradiated with 1,000,000 rads and preserved in liquid nitrogen for use in immunization experiments designed to determine the effect of modifications in the spacing and number of vaccine-adjuvant injections, as well as to determine the effect of a smaller challenge inoculum. As in the previous vaccine-adjuvant experiment, the 1 ml vials of preserved irradiated treponemes were thawed the day of inoculation. They were then pooled to give 20 ml of a treponemal suspension containing 4.0 X 10^9 organisms per ml, and combined with an equal volume of 4% sodium alginate - 0.67% calcium gluconate containing 0.4% phenol as a preservative. The morphology of the treponemes in adjuvant by darkfield microscopy was excellent, and no significant change in the degree of motility was noted. Fifteen rabbits were immunized intramuscularly with 4 liquid nitrogen-preserved, γ-irradiated (1,000,000 rads) *T. pallidum* vaccine doses administered, at 4-week intervals, with an equal volume of alginate-gluconate adjuvant. Each rabbit received $\sim$4 X 10^9 treponemes per dose (2.2 ml) for a total of $\sim$16 X 10^9 organisms. No significant weight loss was observed among any of the vaccinated animals during or after vaccination. Darkfield examination of the tested 3-1/2 weeks after each of the first 3 injections and one week after the last injection was negative suggesting the non-infectivity of the vaccine doses. As a further check on infectivity, a single popliteal node and testis were removed from each of the animals one week after the last immunizing injection and inoculated into the testes of serologically non-reactive, normal rabbits. None of the recipient animals showed evidence of infection during the 6-month observation period as measured by negative darkfield examination and TPI tests.

Four weeks after the last immunizing injection, the 15 vaccinated rabbits were challenged at each of 4 sites with 474 *T. pallidum*, Nichols strain, per site. No evidence of immunity to challenge was noted; typical lesions appeared at each site in both the challenged vaccinated and control animals, with no significant difference in the incubation periods. Thus, while γ-irradiation with 1,000,000 rads appears to effect complete inactivation of *T. pallidum* without DNA repair occurring during liquid nitrogen storage, the evidence strongly suggests that irradiation at this level damages the immunogens responsible for stimulating host resistance. Despite the fact that relatively few infectious treponemes may appear in a vaccine γ-irradiated with 650,000 rads and preserved in liquid nitrogen, it remains essential to carry out further experiments with vaccine prepared in this manner employing modifications in the spacing and numbers of vaccine-adjuvant injections; it is equally important to determine whether a relatively small challenge inoculum will clearly reveal the presence of a complete immunity among animals vaccinated in this manner. Thus, freshly isolated *T. pallidum*, Nichols strain, were γ-irradiated with 650,000 rads, and processed as previously described and preserved in liquid nitrogen. The 1 ml vials of preserved irradiated treponemes were thawed the day of inoculation, and pooled to give 18 ml of a treponemal suspension containing 4.3-5.0 X 10^9 organisms per ml, and combined with an equal volume of 6% sodium alginate - 0.67% calcium gluconate adjuvant containing 0.4% phenol as a preservative.

The morphology of the treponemes in adjuvant by darkfield microscopy was excellent, and no significant change in the degree of motility was noted. Twelve rabbits were immunized intramuscularly with 4 liquid nitrogen - preserved, γ-irradiated (650,000 rads) *T. pallidum* vaccine doses administered, at 4-week intervals, with an equal volume of the adjuvant. Each rabbit received 4.3 to 5.0 X 10^9 treponemes per dose (2.4 ml) for a total of 18.7 X 10^9 organisms. No significant weight loss was observed among any of the vaccinated animals during or after vaccination. Darkfield examination of the testes 3½ weeks after each of the first 3 injections and one week after the last injection were negative, suggesting the non-infectivity of the vaccine doses. As a further check on infectivity, a single popliteal node and testis were removed from each of the

12 animals one week after the last immunizing infection and inoculated into the testes of serologically non-reactive, normal rabbits. None of the 9 surviving recipient rabbits showed evidence of infection during the 6-month observation period, as measured by negative darkfield examination and VDRL tests (TPI and FTA-ABS tests have not been carried out as yet). Five weeks after the last immunizing injection, the surviving 10 vaccinated and 12 control rabbits were challenged at each of 4 sites with 542 T. pallidum per site. No evidence of significant immunity was noted; typical lesions appeared in both the challenged vaccinated and control animals, with no significant difference in the incubation periods.

Thus, it appears that the failure to note an enhancing degree of protection in this experiment utilizing an additional injection of liquid nitrogen-preserved vaccine-adjuvant and a relatively small challenge inoculum is a reflection of either a) immunogen damage during the liquid nitrogen storage processing procedure, b) immunogen damage during storage in liquid nitrogen, c) the relative ineffectiveness of the alginate-gluconate adjuvant, and/or d) the release of soluble surface immunogen(s) into the serum-saline-glycerol supernatant during preparation of the treponemes for liquid nitrogen storage. The latter theory is consistent with the fact that freshly-isolated T. pallidum contains an "outer coat" at its periphery thought to be associated with virulence and which is lost rapidly "in vitro" upon aging (Miller, 1973). In the preparation of T. pallidum suspensions for liquid nitrogen storage, the 66 ml of γ-irradiated treponemes in 50% normal rabbit serum-saline containing 15% glycerol are centrifuged at 19,000 X g for 30 minutes and re-suspended in 1 ml of the supernatant; the remaining supernatant is frozen at -20°C. It is conceivable that during either the irradiation or centrifugation process, soluble surface immunogen(s) detach from the organisms into the surrounding fluid. Indeed, it is possible that the complete immunity demonstrable with freshly-isolated, γ-irradiated T. pallidum may have been due to the injection of soluble surface immunogen(s) contained in the suspensions injected the day of preparation without high-speed centrifugation and liquid nitrogen storage. By this procedure, each of the animals received a total of 60 ml of fresh immunogenic suspensions, while in the experiments carried out with liquid nitrogen-preserved treponemes, each rabbit received a maximum of 4.8 ml of preserved suspensions which may have contained inadequate amounts of soluble immunogen(s). Thus, before initiating attempts to produce acquired resistance with irradiated, preserved *T. pallidum* suspensions utilizing other adjuvants, experiments will be carried out to determine whether protection can be achieved with the serum-saline-glycerol supernatant fluids (with and without complete Freund's adjuvant) obtained from the γ-irradiated (650,000 rads), liquid nitrogen-preserved treponemes employed in the previously described studies.

2. Antibody Response

Inasmuch as VDRL, TPI and FTA-ABS antibodies are important in the diagnosis and control of syphilis, the finding that they develop during the 37-week intravenous immunization process with freshly isolated irradiated treponemes and persist in some animals for at least one year after vaccination is of great significance, and points to the serious restrictions which could be imposed upon a human vaccine with the same antibody-producing capacity (Miller, 1973). Thus, is it interesting to note that while both VDRL and FTA-ABS antibodies developed following the intramuscular and intravenous immunization of rabbits with liquid nitrogen-preserved treponemes (650,000 rads) with or without adjuvants, TPI antibody failed to develop in any of the 20 animals vaccinated by the intramuscular route with or without adjuvants, (including the animal which received infectious treponemes), and in only 3 of the 9 rabbits vaccinated intravenously. Further, no evidence of a TPI anamnestic response was observed in any of the experiments at 3-days or 1-month following challenge. Similar results were obtained among the 15 rabbits vaccinated with T. pallidum γ-irradiated with 1,000,000 rads and administered intramuscularly with adjuvant as described. Each of the 10 rabbits vaccinated intramuscularly with 4 spaced doses of γ-irradiated (650,000 rads), liquid-nitrogen-preserved treponemes developed VDRL antibody (FTA-ABS and TPI antibody determinations have not been carried out, as yet). There is the possibility that VDRL antibody may have developed in response to rabbit tissue present in the vaccine rather than to the treponemes, per se, particularly in light of findings by Smibert that cardiolipin is present in rabbit testicular tissue and absent from T. pallidum, Nichols strain (Personal Communication). Such findings lend further impetus to the necessity for studies relating to both the purification and "in vitro" cultivation of T. pallidum.

3. Degree of Heterologous Acquired Resistance

The data of Turner and Hollander (1957) suggest that rabbits infected with the Nichols strain of T. pallidum exhibit a lesser degree of immunity to re-infection with some heterologous rabbit-adapted human strains as compared with the homologous strain. Further evidence for possible protective antigenic dissimilarity among strains emanates from studies in this laboratory in which it has been shown that (a) a lipopolysaccharide antigen from the avirulent cultivatable T. reiteri is shared by the rabbit-adapted Nichols strain of T. pallidum but not by human strains (Miller, DeBruijn, Bekker and Onvlee, 1966), (b) an ultracentrifugally homogeneous polysaccharide isolated from the Nichols strain reacts with homologous rabbit but not human anti-syphilitic sera (Miller, Bekker, DeBruijn and Onvlee, 1969), and (c) rabbit anti-syphilitic sera prepared against the rabbit-adapted Utrecht strain of T. pallidum and absorbed with an ultrasonic lysate prepared from the homologous strain, show reactivity in the presence of Nichols strain lysate antigen (Miller, Bekker, Onvlee and DeBruijn, - unpublished data). Of critical importance, then, is the necessity for determining whether animals immunized with and resistant to the Nichols strain of T. pallidum are also immune to heterologous rabbit-adapted human strains. Such studies are predicated upon the successful achievement of acquired resistance employing whole, γ-irradiated T. pallidum, Nichols strain, or soluble surface immunogen(s) as a vaccine in a practical injection schedule.

II. MECHANISM(S) OF THE IMMUNE RESPONSE IN EXPERIMENTAL AND HUMAN SYPHILIS (Objective No. 2)

A. Humoral Mechanism(s)

Although no evidence was available (prior to 1972) to suggest that humoral mechanism(s) of resistance were operative in experimental and human syphilis (Eagle and Fleischman, 1948; Magnuson, Thompson, and McLeod, 1975; McLeod and Magnuson, 1953; Magnuson, Thomas, Olansky, Taplan, DeMello, and Cutter, 1956; Miller, Fazzan, and Whang, 1963; Miller, Whang, and Fazzan, 1963; Miller, 1973), preliminary experiments were initiated in this laboratory in 1971 in which the methods of passive protection and combined "in vitro"-"in vivo" neutralization were utilized in an effort to reveal humoral expressions of resistance in the experimental model. The success in elucidating a role for humoral antibody in experimental syphilis using these methods has prompted their application to the human disease in an effort to reveal an operative antibody mechanism.

1. Experimental Syphilis

a. Passive Protection

The experimental evidence which appeared to suggest that passive protection could be convincingly demonstrated only by continuous daily intravenous injections of $\sim$ 10 ml of immune rabbit serum (IRS) has been detailed in Renewal Contract 6/24/74. A definitive experiment based upon this rationale was designed in which each of 5 serologically non-reactive, normal rabbits were inoculated intravenously with 3 ml per Kg body weight ($\sim$ 10 ml) pooled IRS freshly obtained from rabbits with a T. pallidum infection of 12 months duration; the pooled sera were injected at 4 hours prior to intradermal challenge with 1.1 X 10^3 T. pallidum at each of 4 sites, and daily thereafter for 37 days. At the same time, fresh normal rabbit serum (NRS) and saline were injected into each of 5 rabbits under the same conditions and for the same length of time. The protection afforded by the immune serum was unequivocal (Bishop and Miller I, 1976). As shown in Table II, typical lesions developed at all challenge sites on rabbits in the NRS and saline recipient control groups after an average incubation period of 13.7 and 13.1 days, respectively; 35 of the 40 lesions ulcerated in an average time of 28.9 and 25.3 days after challenge. All of the lesions were typically erythematous, indurated, and raised. Motile treponemes were demonstrable in aspirates from 34 of the 40 lesions. Healing of the lesions was complete in 53.0 ± 9.7 days in the NRS control rabbits and in 48.8 ± 7.3 in the saline control animals. Passive transfer of NRS did not influence the course of lesion development; the severity of the lesions, the incubation period, the time required for ulceration, and the duration of the lesions were the same in both the NRS and the saline recipient groups.

In contrast, the lesions present in the IRS-injected rabbits developed at 17 of 20 challenge sites, required 41.5 ± 16.3 days incubation, and were non-ulcerative. They measured 12 mm or less in diameter, were pale and flat with irregular borders, and were darkfield negative. The lesions persisted only 13.5 ± 8.5 days as compared to a duration time of 39.2 ± 9.6 and 35.6 ± 6.6 days respectively in NRS and saline recipients.

The wide diversity in the incubation periods and duration of lesions among the "test" animals is a reflection of 2 different patterns of lesion development. Two animals developed, during the administration of serum, relatively small atypical lesions 12 mm in diameter. In one rabbit, the lesions appeared on day 14 at 3 of the 4 challenge sites, disappeared on day 17, reappeared on day 23, and again disappeared on day 26 for the

Table II. The Response to Treponema pallidum Challenge of Rabbits Passively Immunized by the Intravenous Route[a]

		LESIONS										
		Development				Ulceration		Duration (Days)			Darkfield	
		Incubation Period (Days)		Lesions		Ulcerative Lesions					Positive Lesions	
Preparation	Number of Rabbits	Mean[b]	Range	Total Sites	Character	Total Lesions	Time to Ulceration (Days)	Mean[b]	Range	Time to Complete Healing	Total Lesions	
IRS[c]	5	21.5 ± 16.3[f]	14-57	17/20	Atypical[d]	0/17	-	13.5 ± 8.5	3-24	35.5 ± 22.5	0/17	
NRS[c]	5	13.7 ± 1.1[g]	12-15	20/20	Typical[e]	18/20	25.9 ± 5.0	39.2 ± 9.6	28-56	53.0 ± 9.7	18/20	
Saline	5	13.2 ± 1.7	10-15	20/20	Typical	17/20	25.3 ± 3.3	35.6 ± 6.6	27-50	48.9 ± 7.3	15/20	

[a] Rabbits were injected daily for 37 days with 3 ml/kg body weight and challenged 4 hours after the initial injection with 1.1 X 10^3 T. pallidum, Nichols strain, at each of 4 sites on the shaved backs.

[b] Mean ± Standard Deviation.

[c] IRS = Immune Rabbit Serum; NRS = Non-immune Rabbit Serum.

[d] Atypical lesions are pale, flat, irregular, non-ulcerative, and darkfield negative.

[e] Typical lesions are erythematous, indurated, raised, and ulcerative.

[f] $p < 0.05$, Mann-Whitney U-test.

[g] Not Significant.

for the remainder of the observation period. The second animal showed lesions at each of the 4 sites on day 31 with disappearance on day 34 and no recurrence. The remaining 3 rabbits failed to develop lesions until day 51, or 25 days after IRS injection was terminated. Pale, atypical lesions first appeared on these animals at 10 or 12 sites between days 51 and 57 and persisted for 20 days. Prior to their appearance, all lesions had completely healed in 7 of the 10 control animals.

Thus, the results demonstrate conclusively that passive transfer of a relatively large quantity of immune serum daily over 37 days confers to normal rabbits resistance to challenge with T. pallidum, Nichols strain. This resistance is manifested in the marked delay in lesions development and profound decrease in the severity of the lesions.

In an effort to determine whether the delayed, atypical, non-progressive healed lesions in the passively immunized rabbits represented only localized infection, or whether dissemination and intracellular residence had indeed occurred, the lymph nodes, spleen, liver, and testes were removed and inoculated intratesticularly into serologically non-reactive recipient rabbits. Dissemination and the possibility of intracellular residence following challenge was confirmed by the development of darkfield positive lesions in 3 of the 4 surviving recipient animals.

Thus, the results strongly suggest that the use of relatively large amounts of immune serum produces an initial and continued level of "protective" factor(s) in recipient rabbits which contributes to treponemal destruction upon challenge (Bishop and Miller I, 1976). Indeed, the protective effect of large doses of immune serum has since been confirmed by other investigators (Sepetjian, Salussola, and Thivolet, 1973; Turner, Hardy, Newman, and Nell, 1973; Perine, Weiser, and Klebanoff, 1973; Weiser, Erickson, Perine, and Pearsall, 1976). In an effort to determine whether passive immunization could achieve antibody levels present in donor serum, TPI determinations were carried out as markers. The tests were performed on sera obtained from the passively immunized rabbits (1) day after the last serum or saline injection (day 38) and (2) the day of sacrifice (day 212). The data are shown in Table III (Bishop and Miller I, 1976).

TPI reactivity of the donor immune serum pools ranged from 1:42 to 1:928 with a mean titer of 1:429, while the donor non-immune serum pools were non-reactive at the time of injection into the recipient rabbits.

On day 38, the TPI reactivity of those rabbits that received IRS ranged from 1:143 to 1:303 with a mean titer of 1:228. In contrast, those animals that received NRS and saline exhibited values ranging from Non-Reactive (NR) to 1:85 (mean 1:24) and from NR to 1:40 (mean 1:15) respectively, thus suggesting that the antibody in the IRS recipients was derived primarily from the donor immune serum pools; further evidence was provided by the finding that 6 of the 10 control animals had no TPI antibody. It is equally apparent that the mean TPI titer of the passively transferred serum pools was higher than that of the recipient rabbit sera.

It was of interest to note that at day 212 which represents 7 months following challenge and 6 months following the last serum of saline injection, the TPI reactivity of the IRS-recipient rabbits had either decreased or remained at approximately the same level; in contrast, the control animals exhibited significantly increased mean titers. Inasmuch as passively transferred antibody would not be expected to persist in the recipient host for 6 months, it seems apparent that the TPI antibody response exhibited by 3 of the 4 surviving IRS recipient animals is a reflection of the immunogenic stimulation provided by the organism. Conversely, the complete disappearance of TPI antibody from IRS-recipient rabbit No. 2 suggests an absence of infection by _T_. _pallidum_.

Table III. Comparative TPI Antibody Levels in Passively[a] Immunized Rabbits Challenged with *Treponema pallidum*

Preparation Injected	TPI[b] Passively Transferred Serum Levels: Mean[c]	Range	Rabbit Number	Recipient Rabbit Serum: Day 38[d]	Day 212[e]
IRS	429 ± 263	64-928	1	303	297
			2	154	NR[f]
			3	336	14
			4	205	263
			5	143	No specimen[g]
			Mean[c]	228 ± 87	139 ± 156
NRS	NR	NR	6	33	≥ 704[h]
			7	NR	≥ 704
			8	85	≥ 704
			9	NR	≥ 704
			10	NR	189
			Mean[c]	24 ± 37	601 ± 230
Saline	-	-	11	40	≥ 704
			12	NR	297
			13	NR	No specimen
			14	33	≥ 704
			15	NR	440
			Mean[c]	15 ± 20	536 ± 202

[a] Each of 5 rabbits received immune rabbit serum (IRS), non-immune rabbit serum (NRS), or saline; rabbits were injected daily for 37 days with 3 ml/kg. body weight and challenged 4 hours after the initial injection with 1.1 X 10[illegible] *T. pallidum*, Nichols strain, at each of 4 sites on the shaved backs.

[b] TPI = *Treponema pallidum* Immobilization; reciprocal of the highest dilution which immobilizes 50% of the organisms.

[c] Mean ± Standard Deviation.

[d] Represents 38 days after challenge and 1 day after the last injection of serum or saline.

[e] Represents 212 days after challenge and 175 days after the last injection of serum or saline.

[f] NR = Non-reactive.

[g] Rabbit died from unknown causes before day 212.

[h] Highest dilution tested was 1:704.

It seems evident from these data and from those of the preliminary experiments that the successful demonstration of significant passive protection is predicated to a large extent upon (1) relatively large volumes of immune serum, (2) fresh serum, and (3) daily serum injections. It is probable that the humoral factor(s) involved are immunoglobulins rather than lymphokines, although the participation of the latter cannot be unequivocally excluded (Bishop and Miller I, 1976).

The failure of passive immunization to effect complete protection is evident not only from the eventual appearance of atypical lesions but also from the fact that dissemination occurred in 3 of the 4 surviving IRS-recipient animals. This failure may have been due to the fact that either (1) an insufficient amount of serum was injected before challenge, and hence the circulating and extravascular levels of the immune factor(s) were too low at the time of challenge, or (2) the dilution of donor serum upon the injection resulted in a reduction of humoral immune factor(s) below the concentration necessary to effect complete protection. If TPI antibody levels can be used as a marker, the finding that the mean TPI titer of the transferred serum pools was higher than that of the recipient animals at the end of the injection schedule lends support to both of these hypotheses.

Complete protection by passive immunization may also be precluded on the basis that the large amount of unpurified, whole serum from allogeneic donors injected into the rabbit recipients may have induced the production of antibody against donor immunoglobulins or other serum factor(s), thus resulting in the inactivation of specific treponemicidal factors present in the immune serum. One cannot exclude the possibility that cell-mediated phenomena play a role in the immune process and therefore complete protection by transfer of immune serum only is not possible; this, of course, presumes that immunoglobulins rather than lymphokines are the sole protective factor(s) present in immune serum (Bishop and Miller I, 1976).

The delay in lesion development until after discontinuance of the serum treatment has been previously observed (Perine, Weiser, and Klebanoff, 1973; unpublished data, Bishop) and suggests that in some passively immunized animals the pathologic processes which induce the skin lesions do not become operative until serum administration has been terminated. The intracellular location ot T. pallidum demonstrated by Sykes and Miller (1971) offers a mechanism whereby the organism can elude treponemicidal humoral factor(s); extracellular treponemes are exposed to the action of the passively transferred immune humoral factor(s), but those which entered cells shortly after challenge may become inaccessible to serum factors. Upon termination of immune serum injections, subsequent decline in the level of the circulating and extravascular immune factor(s) allows viable intracellular treponemes to survive in an extracellular environment and induce local lesion formation. This may explain why (1) lesions failed to develop in 3 IRS recipients until 17 days after injections were terminated and (2) the duration of those lesions which developed during the time of continuing serum administration was very short (4 days), while those which appeared after the injections were terminated persisted longer (20 days) but still not as long as lesions in the control groups (39 and 37 days).

Although the atypical local lesions and/or delay in the development of such lesions was a reflection of the protection provided by passive immunization, dissemination occurred in 3 of the 4 surviving IRS-recipient animals. It was of interest to note that at this time TPI antibody levels either remained the same or decreased in contrast to the significantly elevated TPI titers of the non-protected control animals. This seems to suggest that (1) the action of the passively transferred immune serum had reduced the numbers of treponemes available for stimulation of TPI antibody production; (2) the disseminated treponemes resided intracellularly and thus provided no stimulus for TPI production; or (3) injection of pre-formed antibody suppressed

B cell stimulation and prevented subsequent TPI antibody production (Bishop and Miller I, 1976).

The demonstration of a role for humoral factor(s) in immunity in experimental syphilis necessitates the elucidation of whether immunoglobulins are the factors operative in the passive protection of rabbits by immune serum. These studies have been planned. In addition, studies have been planned to determine whether similar humoral factor(s) demonstrable by passive immunization are operative in the serum of rabbits vaccinated with either γ-irradiated (Miller, 1973) or time-inactivated (Metzger, Michalska, Podwinska, and Smogor, 1969) T. pallidum.

b. Combined "In Vitro"-"In Vivo" Neutralization

A second approach to the demonstration of humoral mechanisms of resistance is based upon the use of a neutralization technique similar to that employed in studies relating to viral immunity. Turner (1939) first utilized this technique but negated his results on the basis of subsequently acquired knowledge regarding the incubation survival of T. pallidum. The decision to re-explore the use of this method was based upon the development of newer knowledge relating to "in vitro" survival of treponemes. Further, it was felt that the technique not only might provide a tool for elucidating humoral expressions of immunity, but might also allow a quantitative assessment of the development and persistence of the immune response during the course of the experimental disease. Preliminary experiments did, indeed, provide evidence of an operative humoral mechanism(s). Sera were obtained from 2 infected-immune and from non-immune rabbits on the day of the experiment. A suspension of T. pallidum containing 10^4 treponemes/ml was prepared and added, in equal volumes, to tubes containing each of the immune and pooled non-immune sera. The mixtures were then equilibrated with 95% N_2 - 5% CO_2 and placed in the 34°C incubator. After 4 and 16 hours respectively, 0.2 ml samples of each of the mixtures (containing

1000 treponemes) were inoculated intradermally at each of 4 sites on the shaved backs of normal recipient rabbits for each time schedule. Those sites which received the immune serum-treponemes mixtures incubated for 4 hours developed generally smaller lesions after a 2 to 4 day longer incubation period as compared to those sites injected with the non-immune serum-treponeme suspension. Those sites which received immune serum-treponeme mixtures incubated for 16 hours failed to develop lesions during the 56-day observation period as compared to the non-immune serum-treponeme control sites where lesions development occurred in 14 to 18 days. This clear demonstration of a treponemicidal factor(s) in immune serum led to a more definitive experiment designed to (1) confirm the initial findings, (2) elucidate the role of a heat-labile factor(s), if any, in the reaction, and (3) correlate the development and persistence of immunity to challenge with quantitative levels of the "neutralizing" factor(s). Fifty rabbits were injected intratesticularly with T. pallidum, Nichols strain, and the etiology of the resulting orchitis confirmed by darkfield microscopy. Intradermal challenge of a representative number of rabbits with T. pallidum at each of 4 sites on the shaved back at 11 days, 1 month, 3 months, 4½ months, 7 months, 17 months, and 24 months post-infection revealed a developing but incomplete immunity at 11 days with solid resistance appearing at 3 months and persisting for at least 2 years following infection. The animals were bled 2 days prior to or shortly after challenge and the sera frozen for use in the neutralization studies. The basic technical design was modified somewhat from the preliminary experiment in that 1 volume or treponemal suspension was combined with 9 volumes of pooled test or control serum, either undiluted or diluted and either unheated or heated at 56°C for 30 minutes, to give a final concentration of 10^4 treponemes/ml in the preparation. After equilibration under 95% N_2 - 5% CO_2 and incubation at 34°C for 4 or 16 hours depending upon the experiment, 0.1 ml of the treponeme-serum mixtures, each containing 1000 T. pallidum were injected intradermally into the shaved backs of 5 serologically non-reactive rabbits to determine virulence. The preliminary observations that specific treponemal inactivation by immune serum factor(s) commenced within a 4-hour incubation period and is complete within 16 hours was confirmed (Bishop and Miller II, 1976). As shown in Table IV, rabbits inoculated with treponemal suspensions incubated 4 hours with unheated 4½ month IRS developed typical lesions after a 2 to 7 day delay in the incubation period compared to the control sites, while those animals injected with the same unheated IRS treponeme mixtures but incubated for 16 hours failed to develop lesions at any of the inoculated sites, in contrast to the appearance of lesions in 11 to 15 days at all sites inoculated with unheated NRS treponeme mixtures similarly incubated. The participation of heat-labile serum factor(s) in the reaction was shown convincingly by the development of typical lesions within 15-21 days at those sites inoculated with heated 4½ month IRS treponeme mixtures incubated for 16 hours. As shown in Table V, the inactivation of treponemes by IRS begins within 8 hours of *in vitro* incubation, is significant but incomplete in 12 hours, and becomes complete within 16 hours. "Aging" the treponemes in unheated NRS, under the same conditions of incubation, for 4 hours followed by 4 hours of incubation with IRS, effected complete inactivation. In contrast, the same suspension incubated 8 hours with IRS alone produced lesions at 5 of the 10 inoculated sites. "Aging" of the treponemes for 8 hours followed by 4 hours of incubation with IRS also effected complete neutralization, while the same suspension incubated with IRS alone for 12 hours produced a lesion at 1 of the 5 inoculated sites.

Table VI shows that the injection of rabbits with IRS-treponeme mixtures incubated with and without 100 μg/ml of lysozyme for 4 to 8 hours failed to show significant differences in the time of appearance and in the number of lesions which developed, thus clearly demonstrating that this concentration of added lysozyme does not influence neutralization of *T. pallidum*

Table IV Neutralizing Effect of Immune Rabbit Serum on T. pallidum Incubated In Vitro and Assessed In Vivo in Rabbits[a]

Serum	Incubation Period (hours)	LESIONS							
		Development			Ulceration			Darkfield Positive Lesions	
		Lesions	Incubation Time (days)		Lesions Ulcerated	Time to Ulceration (days)			
		Total Sites	Mean[b]	Range	Total Lesions	Mean[b]	Range	Total Lesions Examined	
IRS[c], unheated	4	9/10	15.3 ± 5.3[e]	13-29	8/9	35.0 ± 1.9	32-39	7/9	
IRS, heated[d]	4	10/10	18.6 ± 6.4[e]	12-29	6/10	32.8 ± 2.9	29-35	5/8	
NRS[c], unheated	4	10/10	11.9 ± 1.3	11-15	10/10	33.1 ± 2.3	29-35	9/10	
NRS, heated	4	10/10	12.1 ± 1.3	11-14	8/10	34.6 ± 1.7	31-37	8/8	
IRS, unheated	16	0/10	-	-	-	-	-	-	
IRS, heated	16	9/10	16.8 ± 1.9[f]	15-21	9/9	39.5 ± 2.8	37-45	5/6	
NRS, unheated	16	10/10	13.3 ± 1.4	11-15	9/10	29.1 ± 1.9	27-32	9/9	
NRS, heated	16	10/10	13.2 ± 1.5	11-16	9/10	28.7 ± 3.7	25-30	6/9	

[a] Five rabbits were inoculated intradermally at duplicate sites on the shaved back with 10^3 T. pallidum in each final mixture.

[b] Mean ± Standard Deviation.

[c] IRS = pooled serum from immune rabbit donors infected 4.5 months (Pool 7, Table V); NRS = pooled non-immune rabbit serum.

[d] 56C for 30 minutes.

[e] $p < 0.05$, Mann-Whitney U-Test.

[f] $p < 0.05$, T-test.

Table V The Kinetics of Neutralization of *T. pallidum* by Immune Rabbit Serum[a]

IRS[b] Pool	In Vitro Incubation Period (hours)			Lesion Development		NRS[b] Pool	In Vitro Incubation Period (hours)			Lesion Development	
	With NRS[c]	With IRS[d]	Total	Lesions / Total Sites	Mean Incubation Period[e] (days)		With NRS[c]	With NRS[d]	Total	Lesions / Total Sites	Mean Incubation Period[e] (days)
7[f]	-	4	4	5/5	19.2 ± 7.2[g]	1		4	4	5/5	15.4 ± 1.1
	-	8	8	5/5	22.0 ± 6.8[g]		-	8	8	5/5	14.4 ± 1.6
	-	12	12	1/5[h]	24.0 ± 0.0		-	12	12	5/5	14.2 ± 1.5
	-	16	16	0/5	-		-	16	16	5/5	15.2 ± 1.3
10[f]	-	4	4	5/5	13.2 ± 1.6[i]	2	-	4	4	5/5	13.2 ± 1.1
	4	4	8	0/5	-		4	4	8	5/5	15.0 ± 4.0
	-	8	8	2/5[h]	26.0 ± 1.4		-	8	8	5/5	13.8 ± 0.8
	-	8	8	3/5[h]	23.7 ± 1.5		-	8	8	5/5	13.6 ± 0.5
	8	4	12	0/5	-		8	4	12	5/5	13.8 ± 0.4
	-	12	12	1/5[h]	24.0 ± 0.0		-	12	12	5/5	14.6 ± 1.1
7	12	4	16	0/4	-	1	12	4	16	4/4	14.5 ± 2.1
	-	16	16	0/4	-		-	16	16	4/4	14.5 ± 2.1

[a] Five rabbits were injected intradermally at each of 8 sites on the shaved back with 10^3 *T. pallidum*, Nichols strain, in the final mixtures. One rabbit died of unknown causes prior to lesion development.

[b] IRS = unheated immune rabbit serum; NRS = unheated non-immune rabbit serum.

[c] Final concentration in NRS = 10^5 *T. pallidum*/ml.

[d] Final concentration = 10^4 *T. pallidum*/ml.

[e] Mean ± Standard Deviation.

[f] Different pools of serum obtained from 4.5-month infected donors.

[g] Not Significant, Mann-Whitney U-Test.

[h] $P < 0.05$, Chi-Square for Goodness-of-Fit.

[i] $P < 0.05$, T-Test.

Table VI Influence of Lysozyme upon In Vitro Incubation Time Required for Neutralization of T. pallidum[a]

IRS[b] Pool	In Vitro Incubation Period (hours)					Lesion Development		NRS[b] Pool	In Vitro Incubation Period (hours)					Lesion Development	
	Without Lysozyme		With 100 µg/ml Lysozyme						Without Lysozyme		With 100 µg/ml Lysozyme				
	NRS[c]	IRS[d]	NRS[c]	IRS[d]	Total	Lesions/Total Sites	Mean Incubation Period[e] (days)		NRS[c]	NRS[d]	NRS[c]	NRS[d]	Total	Lesions/Total Sites	Mean Incubation Period[e] (days)
10[f]	-	4	-	-	4	5/5[g]	18.2 ± 1.6	2	-	4	-	-	4	5/5	13.2 ± 1.1
	-	-	-	4	4	4/5[g]	20.5 ± 1.7		-	-	-	4	4	5/5	13.4 ± 1.5
	4	4	-	-	8	0/5	-		4	4	-	-	8	5/5	15.0 ± [illegible]
	-	-	4	4	8	0/5	-		-	-	4	4	8	5/5	14.9 ± 2.7
	-	8	-	-	8	3/5[g]	23.7 ± 1.5		-	8	-	-	8	5/5	13.5 ± 0.5
	-	-	-	8	8	2/5[g]	25.5 ± 2.1		-	-	-	8	8	5/5	13.3 ± 0.4
7[f]	12	4	-	-	16	0/4	-	1	12	4	-	-	16	4/4	14.5 ± 2.1
	-	-	12	4	16	0/4	-		-	-	12	4	16	4/4	14.5 ± 2.1
	-	16	-	-	15	0/4	-		-	16	-	-	15	4/4	14.5 ± 2.1
	-	-	-	16	16	0/4	-		-	-	-	16	15	4/4	14.5 ± 2.1

[a] Five rabbits were injected at either 8 or 10 sites on the shaved back with 10^3 T. pallidum, Nichols strain, in the final mixtures. One rabbit died of unknown causes prior to lesion development.

[b] IRS = unheated immune rabbit serum; NRS = unheated non-immune rabbit serum.

[c] Final concentration in NRS = 10^3 T. pallidum/ml.

[d] Final concentration = 10^4 T. pallidum/ml.

[e] Mean ± Standard Deviation.

[f] Different pools of serum obtained from 4.5 month infected donors.

[g] Not Significant, Chi-Square for Goodness-of-Fit.

under these test conditions. The absence of lesions at all sites injected with treponemal suspensions incubated 16 hours with IRS or preliminarily "aged" with NRS for 4 or 12 hours prior to the 4 hours incubation with IRS precludes any further conclusions regarding the influence of lysozyme.

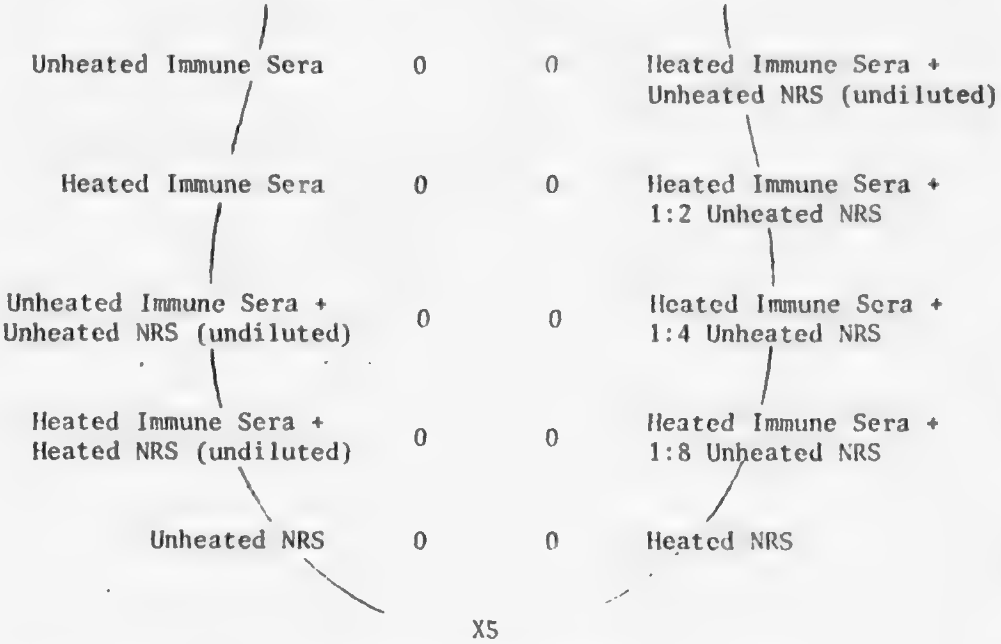
A number of questions regarding the mechanism of inactivation by IRS are raised by these findings. One possibility is that an outer coat or envelope must be removed from _T. pallidum_ to allow exposure of antigenic sites which are susceptible to specific inactivation by immune factor(s). The coat has been suggested to be mucopolysaccharide in nature and therefore susceptible to degradation by lysozyme; pre-incubation with unheated NRS may provide serum lysozyme necessary to effect coat removal. Since 4 hours of incubation with NRS followed by 4 hours of incubation with IRS inactivated the treponemes, incubation for 8 hours with IRS would be expected to similarly accomplish complete inactivation, but failed to do so in the experiments. Loss of lysozyme activity during storage of the IRS may account for this result; however, addition of 100 μg/ml of lysozyme failed to enhance the reaction. These findings contrast with those of Metzger _et al_., who demonstrated the acceleration of serologic reactions by the addition of lysozyme. Although treponemes were not pre-incubated with heated NRS, similar results would be expected if pre-incubation allows removal of an outer coat by serum lysozyme, since the transition temperature for thermal denaturation of lysozyme is well above 56°C (Bishop and Miller II, 1976).

A second possibility is that exposure to IRS alters the envelope or outer surface of the treponemes and prolongs the time required for inactivation. Miller (1973) has suggested that an immunogen present in freshly isolated treponemes and necessary for the development of complete immunity in rabbits may be located in a mucopolysaccharide outer coat. If, indeed, such an antigen exists, it is possible that immune serum contains antibodies which bind to the outer coat and stabilize it against degradation.

It is possible that pre-incubation with NRS removes antiglobulin factors associated with T. pallidum, which have been reported by Logan (1974), and allows accelerated specific inactivation by IRS. This mechanism anticipates an immunoglobulin nature for the immune serum factor(s).

Another possibility suggests an entirely different mechanism for inactivation of the treponemes, namely lysis by the action of the alternative pathway of complement (Mueller-Eberhard, 1975). The components of this system are known to react with polysaccharides, and the sequence of reactions can be activated by immunoglobulin (Ig) aggregates. It may be possible for T. pallidum to bind the complement (C) components which initiate the reaction, inasmuch as a polysaccharide has been isolated from the organism (Miller, Bekker, DeBruijn, Onvlee, 1969). The shorter incubation required for inactivation of the treponemes after pre-incubation with fresh, unheated NRS could be explained by the higher concentration of active C than was present in the IRS after storage. However, this nonspecific mechanism would not be consistent with the data unless it required activation by a component present only in IRS, since NRS alone did not inactivate the treponemes.

Experiments designed to determine whether the heat-labile factor(s) in immune serum which participate in the neutralization of T. pallidum are operative alone or in combination with heat-stable factor(s) during the immune state have been completed; the role of both heat-stable and heat-labile factors has been convincingly demonstrated (Bishop and Miller II, 1976). Immune sera from 3-month and 6-month infected rabbits were heated at 56°C for 30 minutes. Equal volumes of unheated normal rabbit serum (NRS) undiluted and serially diluted to 1:8 with heated NRS were added to the heated immune sera and the neutralization test utilizing 1000 T. pallidum as antigen was carried out as previously described. Control consisting of unheated immune sera, heated immune sera, unheated immune sera + undiluted unheated NRS in equal volumes, heated immune sera + undiluted heated NRS in equal volumes, unheated NRS, and heated NRS were included in the test. Following the 16-hour incubation period, 5 serologically non-reactive rabbits were inoculated intradermally on the shaved back as follows:


Unheated Immune Sera
0
0
Heated Immune Sera + Unheated NRS (undiluted)
Heated Immune Sera
0
0
Heated Immune Sera + 1:2 Unheated NRS
Unheated Immune Sera + Unheated NRS (undiluted)
0
0
Heated Immune Sera + 1:4 Unheated NRS
Heated Immune Sera + Heated NRS (undiluted)
0
0
Heated Immune Sera + 1:8 Unheated NRS
Unheated NRS
0
0
Heated NRS
X5

The results of the experiment are summarized in Table VII.

The data clearly show that the neutralizing property of immune sera is dependent upon the presence of a heat-stable factor(s), presumably immunoglobulin in nature, operative in combination with a heat-labile factor(s) also present in normal rabbit serum (complement?). It is unlikely that the immune serum components are lymphokines and thus indicative of cell-mediated immunity, because of the dependence of the reaction on the non-immune component, presumably C. The exclusive use of the homologous system of T. pallidum, Nichols strain, with sera from infected animals raises the possibility of a non-specific serum component responsible for inactivation. However, since only the heat-labile component is found in NRS, any non-specific mechanism would necessarily require the action of an immune serum component produced or mobilized in response either to a T. pallidum antigen or to the disease process. One such

Table VII Role of Heat-Labile and Heat-Stable Factors in the Neutralization of *Treponema pallidum* by Immune Serum[a]

Serum Preparations Incubated With *T. pallidum*	LESION DEVELOPMENT			
	3-Month Immune Serum		6-Month Immune Serum	
	Lesions / Total Sites	Mean Incubation Period[b] (Days)	Lesions / Total Sites	Mean Incubation Period[b] (Days)
Unheated IRS[c]	1/5	24.0 ± 0.0	0/5	-
Heated[d] IRS	5/5	18.0 ± 1.0	5/5	19.2 ± 1.6
Unheated IRS + Unheated NRS[c] (Undiluted)	0/5	-	0/5	-
Heated IRS + Heated NRS (Undiluted)	5/5	17.0 ± 1.6	5/5	17.6 ± 2.1
Heated IRS + Unheated NRS (Undiluted)	1/5	23.0 ± 0.0	0/5	-
Heated IRS + 1:2 Unheated NRS[e]	5/5	18.2 ± 0.8	5/5	19.0 ± 2.0
Heated IRS + 1:4 Unheated NRS	5/5	16.4 ± 1.3	5/5	17.6 ± 1.9
Heated IRS + 1:8 Unheated NRS	5/5	15.2 ± 1.1	5/5	17.0 ± 2.3
Unheated NRS	5/5	14.8 ± 0.4	5/5	17.0 ± 2.3
Heated NRS	5/5	15.0 ± 0.7	5/5	16.0 ± 3.1

[a] Five rabbits were inoculated intradermally at each of 10 sites on the shaved back with 10^3 *T. pallidum*, Nichols strain, in the final preparations following 16 hours *in vitro* incubation.

[b] Mean ± Standard Deviation.

[c] IRS = immune rabbit serum; NRS = non-immune rabbit serum.

[d] 56C for 30 minutes.

[e] The diluent was heated NRS.

mechanism operative in bacterial destruction, the alternative pathway of complement, has been discussed. Studies to determine whether the heat-stable factor(s) are, indeed, immunoglobulin in nature as well as studies to determine if neutralizing factors are operative, and their nature, in vaccinated animals, have been planned.

Of considerable interest was the relatively close qualitative and quantitative correlation between the "neutralizing" effect of the serum and the immune status of the source rabbits during the course of the disease (Bishop and Miller II, 1976). As shown in Table VIII, pooled serum obtained from rabbits 2 days prior to the 11-day challenge in which it was demonstrated that immunity was just developing, failed to exert a treponemicidal effect upon T. pallidum; rabbits inoculated with the unheated 9-day serum-treponeme mixtures developed lesions at every site at approximately the same time as the control sites. Serum obtained from rabbits infected for 1 month, at a time when immunity to challenge appeared complete, showed an incomplete but significant neutralizing effect. Four of the 10 sites inoculated with the undiluted, unheated serum-treponeme mixture failed to show lesions. At 3 months post-infection, when immunity to symptomatic reinfection appeared to be complete, the neutralizing effect increased; 8 of the 10 sites failed to develop lesions. Pooled serum obtained from rabbits 4.5 and 17 months PI, at a time when the persistence of complete immunity to symptomatic reinfection was demonstrable by challenge, either completely neutralized the treponemes or reduced the virulent organisms below the minimum number necessary to produce a visible lesion; none of the 10 sites inoculated with each of these serum-treponeme mixtures developed lesions. The quantitative data, shown in Table IX, seem to support this correlation. There seems little doubt that the relatively high neutralizing end point of sera from rabbits infected for 3, 7, and 17 months accurately reflects their solidly immune state demonstrable by challenge. Thus, it is indeed conceivable that the technique of neutralization may provide a suitable means for

Table VIII Qualitative Relationship Between the Development of Neutralizing Serum Factor(s) and Immunity in Experimental Syphilis[a]

Characterization of Immune Serum Pools			Lesion Development			
	Donors		Lesions	Incubation Period (days)		Demonstrable Neutralizing Effect of Immune Serum on *T. pallidum*
Pool No.	Duration of Infection at Time of Bleeding	Status of Immunity to Challenge at Time of Bleeding	Total Sites	Mean[b]	Range	
1	9 days	Susceptible[c] (Ulcerative, DF +[d])	10/10	14.1 ± 3.3	11-21	None
		Normal Control	20/20	13.9 ± 2.0	11-18	
2	9 days	Partial[c] (Non-ulcerative, DF+)	10/10	14.5 ± 3.8	10-23	None
		Normal Control	20/20	14.2 ± 4.0	11-24	
3	9 days	Partial[c] (Non-ulcerative, DF-[d])	10/10	13.7 ± 2.3	10-17	None
		Normal Control	20/20	14.2 ± 4.0	11-24	
4	9 days	Resistant[c]	10/10	13.1 ± 2.6	11-18	None
		Normal Control	20/20	13.9 ± 2.[illegible]	11-18	
5	1 month	Resistant	6/10	26.7 ± 5.1[e]	19-36	Partial
		Normal Control	20/20	16.3 ± 4.5	11-27	
6	3 months	Resistant	2/15	27.5 ± 0.7	27-28	Complete[f]
		Normal Control	30/30	16.3 ± 4.5	11-29	
7	4.5 months	Resistant	0/10	-	-	Complete
		Normal Control	20/20	16.0 ± 4.2	11-31	
9	17 months	Resistant	0/10	-	-	Complete
		Normal Control	20/20	16.0 ± 4.2	11-31	

---

[a] Ten or 15 rabbits were inoculated intradermally on the shaved back at each of 8 or 10 sites with 10^3 *T. pallidum*, Nichols strain, in the final mixture following 16 hours incubation. Each test preparation was injected at 1 site and each control preparation at 2 sites per rabbit.

[b] Mean ± Standard Deviation.

[c] Determined at 13 days post-infection (see Table II).

[d] DF+ = darkfield positive; DF- = darkfield negative.

[e] $P < 0.05$, T-Test.

[f] See Table VII.

Table IX Quantitative Relationship between the Development of Neutralizing Serum Factor(s) and Immunity in Experimental Syphilis[a]

Characterization of Immune Serum Pools			Immune Serum Pool Dilutions (Reciprocal)										
	Donors		1	2	4	8	16	32	64	128	256	512	
Pool No.	Duration of Infection at Time of Bleeding	Status of Immunity to Challenge at Time of Bleeding	Lesions / Total Sites										Neutralizing Endpoint (NEP)[c]
1	5 days	Susceptible	10/10	5/5	5/5	-	-	-	-	-	-	-	0
2	6 days	Partial	10/10	5/5	5/5	-	-	-	-	-	-	-	0
3	7 days	Partial	10/10	5/5	5/5	-	-	-	-	-	-	-	0
4	9 days	Resistant	10/10	5/5	5/5	-	-	-	-	-	-	-	0
5	1 month	Resistant	4/10	5/5	5/5	-	-	-	-	-	-	-	< 1
6	3 months	Resistant	2/15[b]	2/10[b]	0/10[b]	0/5	1/5	5/5	5/5	9/10	5/5	5/5	16
7	7 months	Resistant	0/5	0/5	0/5	0/5	0/5	0/5	1/5	5/10	5/5	5/5	128
8	12 months	Resistant	0/6[d]	0/6	0/6	0/6	0/6	2/6	1/6	9/9	4/5	5/5	64

[a] In 2 separate experiments rabbits were injected intradermally at 10 or 11 sites on the shaved back with 10^3 *T. pallidum*, Nichols strain, incubated for 16 hours *in vitro* with various dilutions of unheated immune rabbit serum (IRS) in unheated normal rabbit serum (NRS) diluent. Lesions developed at 49 of the 50 control sites.

[b] Combined data from 2 experiments.

[c] Neutralizing Endpoint (NEP) = the highest dilution of immune serum which, when incubated with the treponemes under the conditions of the test system, prevented the development of lesions at 50% or more of the inoculated sites.

[d] One animal died prior to lesions development.

evaluating the immune status of the host. An immune mechanism dependent upon the synthesis of specific anti-treponemal antibodies is consistent with the observed correlation between the development of immunity to challenge in the donor animals and the appearance and level of the neutralizing factor. The development of resistance to challenge precedes the appearance of demonstrable neutralizing factor in the serum. Although only 2 of 10 rabbits were resistant to challenge 11 days post-infection, pooled serum collected from them on day 9 showed no evidence of inactivating treponemes. It is possible but unlikely that the 2 days between bleeding and challenge significantly altered the response to challenge or the level of immune serum factors. Similarly, 8 of the 9 animals challenged at 1 month were resistant to challenge, and their serum showed evidence of partial neutralization. It is possible that the neutralizing factor, presumably antibody, was present concurrently with early resistance but in quantities insufficient to be demonstrated by complete neutralization. However, the level of the factor increases during the course of the disease as shown by the NT and then declines somewhat late in the infection in the manner of a typical response curve.

The design of the experiment allows determination only of resistance to symptomatic reinfection and allows no conclusions regarding the correlation between complete resistance to reinfection and the neutralizing serum factor(s). To determine resistance to asymptomatic reinfection as well would necessitate antibiotic treatment of the animals and a delay before attempting reinfection, during which time the immune response of the animal presumably continues. For this reason, earlier reports of resistance to reinfection at 3 weeks (Magnuson and Rosenau, 1948; Turner and Nelson, 1950) may not accurately represent the status of immunity at the time of treatment, but rather at the time of challenge. Additionally, no information was obtained regarding immunity to greater challenge doses during the early course of the infection in our experiment. Studies along both these lines are being planned.

During the development of immunity in the serum donors, the challenge dose used was 10^3 *T. pallidum* per site, and graded challenge doses up to 10^6 treponemes per site were used only at the conclusion of the experiment. It has been shown that rabbits become resistant to challenge with increasingly larger numbers of organisms as immunity develops (Magnuson and Rosenau, 1948). However, it may not be possible to use increased numbers of treponemes in the neutralization test as a measure of greater resistance. Preliminary observations indicate that undiluted serum pools, which had NLP values as high as 128 in neutralization tests using 10^4 treponemes per ml, failed to completely neutralize suspensions containing 10^6 organisms per ml (Bishop and Miller II, 1976).

The inocula, consisting of 10^5 treponemes per site in this experiment, may have been so large that reduction in numbers of viable organisms was masked. It is also possible that either the incubation time or the concentration of immune factor(s) was insufficient for inactivation of the greater number of organisms. Further, no correlation was found between the motility of the treponemes in these suspensions and their ability to produce lesions. Therefore, one must conclude that a neutralization test using relatively small numbers of treponemes and various dilutions of the serum more accurately measures the immunity of the donor than one in which the number of treponemes is increased.

It would be of great interest to determine the presence of the neutralizing serum factor and its correlation with the status of immunity in rabbits immunized with treponemal vaccines inactivated by γ-irradiation (Miller, 1973) or by aging at 4°C (Metzger and Smogor, 1969). A comparison of the levels of the serum factor and its identification as immunoglobulin(s) in immunized and infected-immune rabbits may contribute to the understanding of the mechanism of induction of immunity by treponemal vaccines. These studies have been planned.

Inasmuch as future experiments relating to immune mechanisms will necessitate the use of graded challenge doses of *T. pallidum*, it becomes important to determine whether the use of such graded doses in the same rabbit affects lesion development by the smaller challenge inocula as compared to the use of single doses in one animal. Thus, 4 groups of 5 rabbits each were injected intradermally at each of 8 sites on the back with inocula containing 10^3, 10^4, 10^5, and 10^6 *T. pallidum*, respectively (graded). Four additional groups of 5 rabbits each were injected at duplicate sites with 10^3, 10^4, 10^5, and 10^6 treponemes, respectively (single). Among the four groups which received the graded doses, the positions of the challenge doses were varied such that 5 rabbits were injected at duplicate sites with each challenge dose at each of the 4 possible relative positions (anterior, posterior, etc.). The results of the experiment as determined through day 35 are shown in Table X. The development of lesions and their mean incubation periods were essentially the same in rabbits injected with graded doses and with single doses of *T. pallidum*. The time from inoculation to ulceration of the lesions was also similar for the two groups. However, the proportion of ulcerative lesions which developed from 10^3 and 10^4 treponemes was reduced in animals inoculated with graded doses. In addition, the proportion of these lesions exhibiting motile treponemes in aspirates observed by darkfield microscopy were somewhat reduced.

The effect of the position of the inoculation sites upon the mean incubation period for the resulting lesions is shown in Table XI. When a single dose was used for all sites, the results were highly uniform and the position of the inoculation site on the back did not influence the incubation period. With graded dose inocula the results were more variable; however, no significant effect due to the position of the inoculation site was observed.

[illegible]

[illegible]		[illegible]			[illegible]			[illegible]
		Total [illegible]	Range	Mean	Total [illegible]	Range	Mean	[illegible]
Single Doses	10^6	40/40	[illegible]	[illegible]	[illegible]/40	[illegible]	[illegible]	[illegible]
	10^5	40/40	[illegible]	6.2	[illegible]/40	[illegible]	17.[illegible]	[illegible]
	10^4	40/40	[illegible]	9.0	[illegible]/40	[illegible]	22.[illegible]	10/10
	10^3	40/40	[illegible]	11.5	[illegible]/40	[illegible]	[illegible]	10/10
Graded Doses	10^6	40/40	[illegible]	[illegible]	[illegible]/40	[illegible]	17.3	40/40
	10^5	40/40	[illegible]	6.1	[illegible]/40	[illegible]-28	20.8	40/40
	10^4	40/40	[illegible]	10.[illegible]	19/40	22-35	25.5	33/37
	10^3	39/40	[illegible]	12.[illegible]	14/40	20-35	[illegible].0	26/31

Table XI. Influence of Position of Inoculation Site on the Rabbit Back Upon the Incubation Period of Lesions

T. pallidum/ Site	MEAN INCUBATION PERIOD OF LESIONS, DAYS									
	Single Dose					Graded Doses				
	Position of Site				Σ^b	Position of Site				Σ^b
	1[a]	2	3	4		1	2	3	4	
10^6	4.4	4.1	4.0	4.2	4.2	4.4	4.2	4.3	4.4	4.3
10^5	6.2	6.2	6.2	6.3	6.2	8.4	5.4	5.7	6.0	6.1
10^4	9.1	9.0	9.0	9.0	9.0	11.5	11.8	10.8	7.8	10.4
10^3	11.6	11.5	11.4	11.4	11.5	13.0	12.3	10.2	13.4	12.3

[a] 1 = Anterior; 4 = Posterior

[b] Σ = Mean of all sites inoculated with a given dose.

The results of the experiment suggested that the incubation period required for lesion development at each inoculation site is independent of influence from other inoculation sites as long as the inoculum contains at least 10^3 treponemes. The later course of lesion development may, however, be influenced by other lesions.

To determine whether lesion development from inocula containing fewer than 10^3 treponemes is influenced by lesions resulting from large inocula, an experiment similar to the one described was initiated, except that doses of 10^6, 10^3, 10^2, and 10 were employed. The results are shown in Table XII. All sites injected with 10^6 _T. pallidum_ first showed evidence of lesion development on day 3. Mean incubation periods of the lesions developing from single dose inoculation and from graded dose inoculation with 10, 10^2, or 10^3 treponemes were compared by the t-test. In each case, the incubation period was _significantly shorter_ for lesions on rabbits injected with graded doses than those with single doses ($P<.05$). In the graded dose series, 10^3 treponemes produced lesions at all of the 40 sites in 8.4 days whereas the single dose mean incubation period was 12.4 days. Similarly, the incubation periods were 12.1 and 15.0 days for 10^2 treponemes and 16.2 and 20.5 days for 10 treponemes. Additional evidence for the influence of the 10^6-inoculum lesion upon the development of the small-inoculum lesions was found in the decreased proportions of ulcerative and darkfield-positive lesions at sites injected with 10^3 or fewer treponemes in the graded dose series. (Statistical analyses of mean time to ulceration and numbers of sites developing lesions have not yet been performed.)

These results provide an interesting comparison to the data obtained in the previous experiment in which no significant effect was observed utilizing inocula of 10^6, 10^5, 10^4, and 10^3 treponemes in a similar protocol. A third experiment in this series to include graded doses ranging from 10 to 10^6 treponemes has been planned.

Table XII. Comparison of Lesion Development in Rabbits Injected with Single Doses or Graded Doses of T. pallidum

T. pallidum/site		LESIONS						
		Development			Characteristics			
		Lesions/ Total sites	Incubation Period, Days		Ulcerative Lesions/ Total Lesions	Time to Ulceration, Days		DF + Lesions/ Lesions Examined
			Range	Mean		Range	Mean	
Single Dose	10^6	40/40	3	3.0	40/40	7-15	9.8	24/33
	10^3	40/40	12-13	12.4	40/40	16-24	20.7	39/39
	10^2	40/40	12-18	15.0	40/40	27-36	28.1	12/12
	10	38/40	19-27	20.5	33/38	27-40	32.9	4/4
Graded Dose	10^6	40/40	3	3.0	40/40	6-17	9.1	35/40
	10^3	40/40	8-11	8.4	21/40	14-38	21.2	5/21
	10^2	40/40	9-19	12.1	9/39	19-40	25.8	1/10
	10	36/40	12-21	16.2	3/36	23-31	21.7	0/4

2. Human Syphilis

a. Passive Protection

Passive protection experiments with human syphilitic sera necessitate the use of recipient rabbits made tolerant at birth to human globulin. These studies are not as yet underway.

b. Combined "In Vitro" - "In Vivo" Neutralization

The results of the neutralization experiments described for the experimental disease have opened up new avenues of approach to the study of possible humoral immune mechanisms in human syphilis, and provide the rationale for determining whether (1) similar heat-labile and/or heat-stable factor(s) are operative in the human disease, (2) differences in the immune status of patients with early, latent, and late syphilis can be distinguished on the basis of qualitative or quantitative neutralization titers, and (3) demonstrable heat-stable factor(s) are immunoglobulin in nature. These exciting studies have been planned in cooperation with Dr. Joshua Siegel, V.D. Control Officer at the Hollywood-Wilshire V.D. Clinic, Los Angeles County Health Department, where blood specimens from patients with syphilis are being obtained. Serum is removed at the UCLA treponemal laboratory and frozen at -76°C for future use in the planned neutralization experiments analogous to those already described for the experimental system.

Preliminary experiments have been initiated to determine the parameters necessary for survival of T. pallidum (as measured by both motility and virulence) in the presence of unheated normal human serum as a necessary prerequisite to the definitive neutralization studies described above. The use of unheated normal human serum as an extraction medium for T. pallidum resulted in motilities of less than 70% established as a criterion for acceptability following incubation periods of less than 10 hours at 34°C under

95% N_2 - 5% CO_2. Poor motility was also obtained at less than 12 hours of incubation when extraction was carried out in Nelson's medium and an amount of unheated normal human serum added to give a final concentration of 90% (as utilized in the experimental rabbit system). However, when unheated normal human serum was added to T. pallidum extracted in Nelson's medium to give a final concentration of 30%, the motility was 70% or greater after 12 hours of incubation at 34^oC under 95% N_2 - 5% CO_2 equilibration. It was interesting to note that satisfactory treponemal survival seemed to be dependent upon a 30-minute aerobic incubation every 2 hours followed by re-equilibration and re-incubation from the initiation of incubation until the final reading at 12 hours. Completed preliminary experiments have suggested that despite satisfactory motility of T. pallidum in the presence of 30% unheated human serum after 12 hours of incubation, lesions failed to develop consistently following intradermal inoculation of such serum-treponeme mixtures, suggesting a treponemicidal effect not correlative with the finding of satisfactory motility. In an effort to explain the discrepancy between motility and the results of the infectivity tests, it was postulated (Phil Hanff - graduate student) that the amount of normal serum per treponeme was greater in the 10^4/ml than in the 10^7/ml suspension. If cross-reacting antibody is the mediator of non-specific treponemicidal activity, one could conclude that the neutralization would be greater in the 10^4 T. pallidum/ml suspension due to the larger amounts of antibody per treponeme. Thus, an experiment was designed in which unheated normal human serum, as well as heated normal human serum and unheated and heated normal rabbit sera as controls, were added to a treponemal suspension to give a final concentration of 30% and a final T. pallidum concentration of 10^7/ml. Following 0, 4, 12, and 14 hour incubation periods, motilities ranged from 85% to 100% dilution of the serum-treponeme mixtures were then made with Nelson's medium, just prior to animal inoculation, to achieve final treponemal concentrations of 10^6, 10^5, 5 X 10^4, and 10^4/ml. For each serum-treponeme mixture at each of their respective incubation times 5 serologically non-reactive rabbits were inoculated intradermally with 0.1 ml to give final T. pallidum concentrations of 10^6, 10^5, 10^4, 5 X 10^3, and 10^3 organisms in the presence of the serum; each animal was inoculated intradermally with the corresponding T. pallidum extract incubated for 12 hours as a further control. Typical lesions developed at every site in the expected incubation time, with no significant differences occurring between the test and control sites; the average incubation periods for all 10^6, 10^5, 10^4, 5 X 10^3 and 10^3 T. pallidum sites ranged from 4.4 to 6.4 days, 7.0 to 8.2 days, 11.0 to 12.2 days, 11.6 to 13.0 days, and 13.6 to 14.5 days respectively. Thus, (1) a satisfactory correlation between motility and virulence appeared to exist, and (2) treponemicidal activity on the part of normal human serum was not demonstrable, strongly suggesting the correctness of the original premise leading to the experiment. The theory was further strengthened by the finding that incubation of unheated normal serum in the presence of 10^6 and 10^5 T. pallidum/ml under the same conditions, resulted in the irregular appearance of lesions, despite motilities of $\geq$85%.

In a preliminary experiment with unheated late latent syphilitic serum, untreated, utilizing the successful parameters described above, a significant effect upon the virulence of T. pallidum was consistently observed at the 10^5, 10^4, and 10^3 sites inoculated with preparations incubated for 14 hours. Of the 5 sites inoculated with each mixture, 1, 1, and 2 developed lesions, respectively; the incubation periods of the lesions were significantly delayed (20, 24 and 28.5 days, respectively, compared to the control sites in which lesions ranging from 7.8 to 10.4, 10.8 to 15.2, and 15.0 to 18.4, respectively, developed at all sites. While these exciting results have established a technique for exploring the relationship of neutralizing antibody to immunity in human syphilis, studies are still continuing in an effort to eliminate the treponemicidal activity of normal human serum so as to allow the use of a 90% final serum concentration in the methodology. It has been shown that a treponemal motility of 84%-98% can be maintained under anaerobic conditions for at least 16 hours when *T. pallidum* is extracted in heated normal human serum and heated normal human serum is added in final concentrations of 10% to 50% (the highest concentration used in the experiment). It was also demonstrated that the addition of unheated normal human sera to the heated normal human serum extract of *T. pallidum* in final concentrations ranging from 10% to 50% resulted in motilities of less than 70% under the same conditions of incubation; further, the decrease in motility was roughly proportionate to the increase in unheated serum concentration. Further, as expected, complement activity as measured by an assay system utilizing amboceptor-sensitized sheep erythrocytes, was proportionately greater as the concentrations of unheated serum increased and motility decreased. Thus, the evidence is highly suggestive that heat-stable treponemicidal factor(s) (immunoglobulins?) in concert with heat-labile factor(s) (complement?) present in normal human serum, inactivate *T. pallidum*.

Preliminary studies have already shown that treponemal motility in the presence of unheated normal human serum absorbed with *Treponema phagedenis var reiteri*, and in 90% final concentration was 86% after anaerobic incubation for 13.5 hours; this was in contrast to the 90% unabsorbed serum-treponeme mixture in which the motility was 24% after 4 hours incubation. These data strongly suggest that the heat-stable treponemicidal factor(s) is a cross-reactive treponemal group antibody elicited by host-indigenous non-pathogenic treponemes. Inasmuch as absorption in this manner might allow the use of maximum concentrations of unheated human sera in the neutralization assay, these studies are continuing. The results will determine which neutralization methodology will be employed to determine whether (1) heat-labile and/or heat-stable factor(s) are demonstrable and thus operative in latency, (2) differences in the immune status of patients with early, latent, and late syphilis can be distinguished on the basis of qualitative or quantitative tests, and (3) demonstrable heat-stable factor(s) are immunoglobulin in nature.

B. Cell-Mediated Mechanisms

As indicated in Renewal Application No. 4 (6-25-75) the demonstration that cell-mediated phenomena may participate in the development and persistence of acquired resistance in both experimental and human syphilis has been hampered for the most part by the lack of satisfactory and specific methodology which would allow the performance of experiments from which definitive and unequivocal conclusions could be drawn. A number of confusing reports have appeared in the literature attempting to postulate either a role for or the suppression of cellular immune mechanisms in the human disease based upon blast transformation, or leukocyte migration utilizing either non-specific antigens of T. pallidum contaminated with rabbit testicular tissue as mitogens (Mezzadra, Sapuppo, Lazzaro, and Buzzone, 1969; Janot, Grandidier, Pupil, Thomas, Beurey, and La Vergne, 1971; Fulford and Brostoff, 1972; Musher, Schell, and Knox, 1974).

Since 1973, this laboratory has been concerned with the development and application of reliable techniques for elucidating cell-mediated immune mechanism(s) in the experimental and human disease. Specifically, efforts have been directed toward (a) a measurement of direct cytotoxicity of immune lymphocytes upon T. pallidum and (b) attempted alteration of the immune response and/or enhancement of susceptibility in rabbits with goat anti-rabbit thymocyte globulin.

1. Human Syphilis

a. Direct Cytotoxicity of "Immune Lymphocytes" upon "T. pallidum" Nichols Strain

Determination of the cytotoxic effect of immune lymphocytes upon T. pallidum is predicated upon the preparation of pure lymphocytes which will remain viable during their interaction with suspensions of virulent and motile T. pallidum without exerting a non-specific, harmful effect upon the treponemes. Conversely, the treponeme suspension must allow the survival of lymphocytes during the interaction period.

Previous experiments were based upon the hypothesis that an atmosphere of 95% N_2 - 5% CO_2 is essential for these experiments, inasmuch as the environment is necessary for T. pallidum survival for 40 or more hours. However, findings by Rathlev at the State Serum Institute in Copenhagen and in this laboratory suggested that the use of McCoy's 5a medium allows both lymphocytes (as measured by trypan blue exclusion) and treponemes to survive under aerobic conditions of incubation at 34-35°C for at least the time necessary for specific interaction to occur. As a result of continual and exhaustive preliminary human experiments, modified parameters for the measurement of cytotoxicity more reliable and reproducible than those previously described have been developed. Relatively pure lymphocytes (90%) were prepared from 29 patients with latent syphilis and from normal control donors as follows:

(1) Heparinized blood diluted 1:2 or 1:3 with saline was layered onto a 9% Ficoll-hypaque gradient and centrifuged at 400 X g for 40 minutes.

(2) The lymphocytes separated at the interface were washed several times with Hank's balanced salt solution; each washing was followed by centrifugation at 400 X g for 10 minutes and resuspension of the pellet in Hank's solution.

(3) After the final washing, the lymphocytes were suspended in McCoy's 5a medium to give a concentration of 5 X 10^6 lymphocytes per ml. Treponemal suspensions were prepared per ml, and combined with the "test" and normal lymphocyte preparations to give a 100 to 1 ratio of lymphocytes to treponemes.

Incubation was carried out aerobically for 5-6 hours at 34°C at which time 90%-95% of the lymphocytes were still viable as measured by trypan blue exclusion. At the end of the incubation period, the suspensions were examined for the percentage of motile treponemes based upon the observation of 25-50 organisms. The motility observed in the presence of normal lymphocytes ranged from 75% to 92%. In contrast, 3 latent syphilitic lymphocyte-treponeme preparations showed only 11 to 15% motility, 5 showed from 22 to 35%, 9 showed from 39 to 55%, and 12 showed from 57 to 70%. These results suggest that cell-mediated phenomena may play a role in the human syphilis immune process; plans for their continuation are being formulated. (A graduate student, Robert Doe, will be involved in these studies.)

2. Experimental Syphilis

a. Direct Cytotoxicity of "Immune Lymphocytes" upon "T. pallidum" Nichols Strain

In an effort to determine whether lymphocyte toxicity is operative during the development and persistence of immunity in the experimental rabbit disease, experiments were initiated to determine the parameters necessary for the aerobic survival of treponemes and rabbit lymphocytes under conditions similar to those described for human model. Relatively pure lymphocytes (_95%) were prepared from normal rabbits as follows:

(1) Heparinized blood (20 units/ml) was centrifuged at 1800 RPM four minutes, the buffy coat removed and placed in a Fisher tube, and centrifugation carried out at 2000 X g for 2 minutes.

(2) The concentrated buffy coat was removed and placed into a Fisher tube containing approximately 0.4 ml anti-P serum and 4 drops of ADP solution.

(3) The red blood cells and platelets were agglutinated by slowly inverting the tube several times and were then pelleted by centrifugation at 1000 X g for 3 seconds.

(4) The supernatant was removed and layered gently onto 0.5 ml of a Ficoll-Hypaque mixture in Fisher tubes and centrifuged at 1000 X g for 2 minutes.

(5) The white interface was removed, suspended in McCoy's 5a medium containing 10% heat-inactivated normal rabbit serum (NRS), and centrifuged at 4000 X g for 1 minute to pellet the lymphocytes.

(6) The lymphocytes were washed 3 times, each washing followed by centrifugation at 3500 X g for 1 minute and re-suspension.

(7) After the final washing, the lymphocytes were suspended in the McCoy's-NRS medium to give the proper final concentration.

T. pallidum suspensions were prepared in Nelson's medium containing 10% heat-inactivated NRS and added in equal volumes to the lymphocyte suspensions to give a 5:1 ratio of treponemes to lymphocytes. *Aerobic* incubation at 34°C resulted in the maintenance of greater than 70% motility of *T. pallidum* for at least 10 hours. Further, the viability of the lymphocytes remained at 95% as measured by trypan blue exclusion. In an attempt to determine whether lymphocyte toxicity is operative during the immune process in the experimental disease, the above parameters were modified to give a 100:1 lymphocyte-treponeme ratio and both immune rabbit lymphocyte-treponeme and non-immune rabbit lymphocyte-treponeme mixtures were inoculated intradermally into the shaved backs of 4 serologically, non-reactive rabbits after aerobic incubation at 34°C for 5 and 10 hours; each animal received 10^5 lymphocytes and 10^3 *T. pallidum*. Typical lesions appeared at the same time at all sites in 12-13 days, thus indicating the absence of a cytotoxic effect upon *T. pallidum* by lymphocytes from immune animals under the conditions of the experiment. In an effort to determine whether a treponemicidal effect of immune lymphocytes is mediated by specific treponemal antibody, experiments were designed in which immune and normal lymphocytes were combined with immune serum and *T. pallidum*.

incubated for 12 or 15 hours, then injected intradermally into serologically non-reactive rabbits, together with appropriate controls. In the experiment in which the lymphocyte/T. pallidum ratio was increased to [illegible] to 1, the number of treponemes inoculated was reduced to 500, the immune serum concentration was 50%, and the incubation time was 15 hours, a partial killing effect was observed by both normal and immune lymphocytes in the presence of heated immune serum, the ability of normal lymphocytes to kill T. pallidum in such a system is not surprising in light of a similar effect noted upon tumors. A similar experiment utilizing serial dilutions of immune serum ranging from 1:4 to 1:1024 in combination with both normal and immune lymphocytes (lymphocyte/T. pallidum ratio = 100 to 1, T. pallidum concentration = 40,000/ml, incubation time = 10 hours), showed no evidence of antibody-dependent cellular killing of T. pallidum, lesions developed at all sites 13 days following intradermal inoculation.

Based upon the premise that immune lymphocytes must be preliminarily stimulated or "primed" before a demonstrable cytotoxic response can be evoked, a preliminary experiment was carried out in which both normal and immune lymphocytes were preliminarily incubated aerobically at 34°C with T. pallidum γ-irradiated with 650,000 rads for 21 hours, followed by the addition of virulent T. pallidum and continued incubation for 0, 5, and 10 hours. Following each incubation time, serologically non-reactive rabbits were inoculated intradermally at each of 2 sites with each preparation. The lymphocyte/T. pallidum ratio was 100 to 1 and the number of T. pallidum inoculated per site was 1000. The results were highly encouraging. Lesions failed to develop at either of the 2 sites inoculated with "primed" immune lymphocytes and T. pallidum incubated for 10 hours, in contrast to the development of typical lesions at both the sites inoculated with similar preparations incubated for 0 and 5 hours and at the sites inoculated with "primed" normal lymphocytes and T. pallidum incubated for 0, 5, and 10 hours. Experiments are underway and continuing in an effort to confirm and extend these findings utilizing varying ratios of stimulator (γ-irradiated T. pallidum) to killer (lymphocytes) and killer to target (virulent T. pallidum) and extended "priming" and incubation times.

b. Alteration of Immunity and/or Enhancement of Susceptibility Utilizing Goat Anti-Rabbit Thymocyte Globulin (ATG)

The use of ATG as an immunosuppressive agent causing lymphocyte depletion has been used in numerous experiments in an effort to demonstrate a cellular mechanism(s) of resistance operative in infectious diseases. The preparation, characterization, availability and plans for its definitive use have been described in Annual Report No. 2. As a necessary prelude to determine whether the intravenous administration of 0.6 ml and/or 1.2 ml of ATG/kg body weight would result in rabbit lymphocyte depletion as measured by the results of rabbit skin allografts. Rabbits injected with either unheated or heat-inactivated ATG utilizing either dose died after the first or third injection. Death was attributed to the fact that (1) the ATG may not be specific for lymphocytes alone and is attacking other tissues as well, or (2) the dose was overwhelming, thereby causing "cytotoxic anaphylaxis."

Rabbits were given a total of 8 injections over a 26-day period at 3 or 4 day intervals with two different lesser doses of heat-inactivated ATG (0.6 ml and 1.2 ml total per injection without regard to body weight) in order to determine a non-lethal dosage capable of effecting lymphocyte depletion. Inasmuch as the animals survived, an additional experiment was initiated with 0.2 ml and 0.3 ml doses of heat-inactivated ATG per Kg body weight utilizing the same injection schedule. Skin allografts were carried out after the third injection; no significant difference in the time of rejection of the "test" and "control" grafts by the animals was noted, suggesting the possibility that CMI impairment did not occur. (Autografts used as technical [illegible] were [illegible]. In light of these data, 3 rabbits were given [illegible] intravenous injections of 0.3 ml of heat-[illegible] VTC and 3 rabbits similarly injected with normal [illegible] ([illegible]), for a total of [illegible] days. Skin allografts were [illegible] after the third injection. One of the 3 [illegible] which received VTC showed a delay in rejection, suggesting [illegible]. On the basis of these data, preliminary studies were carried out in which 3 groups of serologically-[illegible] rabbits (3/group) were injected intravenously with 0.3 ml of VTC, [illegible] and saline, respectively, every day throughout the course of the experiment. One day after initiation of the [illegible], each rabbit was challenged intradermally at [illegible] sites with 10^6, 10^5, 10^4, and 100 T. pallidum. No significant differences were observed in lesion development between animals [illegible] with VTC and the control animals. Further studies relating to specificity and [illegible] of VTC are planned for the [illegible] future.

III. [illegible] IN VITRO [illegible] CULTIVATION OF T. PALLIDUM, NICHOLS STRAIN (Objective No. [illegible])

B. [illegible]

[illegible]

[illegible] (Miller, 1973).

[illegible]

techniques using potassium tartrate are used, separation of axial filaments has been noted suggesting that both morphologic and antigenic alterations have occurred (Sykes, unpublished data).

Purification experiments utilizing discontinuous Ficoll density gradients for separation were initiated at the California Hospital Medical Center in cooperation with Dr. John Sykes, who successfully employed this technique for the separation of tumor cells from fibroblasts (Sykes, Whitecarver, Briggs, and Anson, 1970). Preliminary investigations have indicated that the rabbit-adapted Nichols strain of T. pallidum appears to be separable from host tissue by this technique. Intratesticularly-infected rabbits were sacrificed at the height of orchitis development and the testes aseptically removed and segmented. Extraction was carried out in 50% heat-inactivated rabbit serum-saline medium under 95% N_2 - 5% CO_2 equilibration for 1 hour at room temperature. In order to minimize the interfering effects of the fibrinous exudate, the resulting treponemal suspension was diluted 10 times with Eagles minimal essential medium without calcium (MEM) and containing 50% heat-inactivated fetal calf serum. The diluted extract was then strained through 4 layers of gauze to remove further small-particle debris. The supernatant was centrifuged at 19,000 X g for 20 minutes, the resultant pellet re-suspended in 10 ml of the MEM-fetal calf serum solution, and additional clarification carried out by centrifugation at 900 X g for 10 minutes. The suspension was again centrifuged at 19,000 X g for 20 minutes, the supernatant discarded, and the pellet re-suspended in MEM containing Ficoll (density = 1.030). The MEM-Ficoll suspension was layered onto various discontinuous gradients of Ficoll dissolved in MEM and centrifugation carried out at 8,923 X g for 10 minutes at 25°C in a Spinco L-2 HV preparative ultracentrifuge using an SW-50 swinging bucket rotor. Following centrifugation, the various interdensity zones were collected by bottom puncture under direct visual observation and examined by darkfield and electron microscopy; additionally the treponemes from each interface were examined in the FTA-ABS test for antigenic integrity and purity. The cleanest preparations were obtained at the 1.050-1.065 gm/ml interface. Both darkfield and electron microscopy failed to reveal evidence of tissue contamination; FTA-ABS tests employing the gradient treponemes as antigen not only revealed reactivity at the same level as standard, non-purified antigen preparations, but also appeared free of any tissue debris. Of important significance was the finding that gradient treponemes retained their motility, thus lending hope to the possibility that immunogenicity and virulence have also been retained.

Experiments designed to confirm these preliminary findings resulted in the demonstration of "clean" suspensions of treponemes by darkfield microscopy. However, electron microscopy revealed the occasional presence of small amounts of cellular debris.

It seemed apparent that consistently effective separation of treponemes from host rabbit testicular tissue utilizing the density gradient technique necessitated a determination of the true buoyant density of the organism. Studies have been made using linear sucrose gradients with densities ranging from 1.080 gm/ml to 1.280 gm/ml differential centrifugation of the crude testicular extracts. The gradients were centrifuged at various g- forces and for periods of 1 hour and/or 18-20 hours. The experiments so far carried out at 100,000, 50,000 and 20,000 X g have given highly reproducible results. Depending upon loading, three discrete bands were seen and appeared to be due to aggregated organisms in the upper and lower bands with mainly single organisms in the center band. A plot of approximate numbers of single treponemes on a plot of the density of the collected fractions appears to indicate an equilibrium buoyant density in sucrose for T. pallidum, Nichols strain, of 1.190 to 1.193 gm/ml. A detailed electron microscope study of the organisms from the three bands is planned to determine, if possible, the reason for the clumping of the organisms in the upper and lower bands. Preliminary observations have failed to show any gross morphological changes (such as unwinding of axial filaments). Studies are also planned to develop a consistently reproducible Ficoll density gradient technique for separation based upon the recently-determined buoyant density of the organism and to elucidate the ultrastructural nature of the organisms separated in this manner.

B. "In Vitro" Cultivation in Tissue Culture Monolayers

The inability to either culture Treponema pallidum "in vitro" or to separate the organism from the rabbit host testicular tissue in which it is presently propagated, has seriously hampered and made complex those studies relating to (1) metabolism (Wilcox and Guthe, 1966); (2) vaccine development (Metzger and Smogor, 1969; Miller, 1973); (3) serologic specificity in relation to diagnosis (Miller, 1975); (4) mechanism(s) of resistance (Miller, 1973); (5) antigenic structure (Miller, DeBruijn, Bekker and Onvlee, 1966; Miller, Bekker, DeBruijn and Onvlee, 1969); and (6) physico-chemical make-up (Miller, DeBruijn, Bekker, and Onvlee, 1969). While some limited knowledge has and will become available through studies utilizing tissue-contaminated treponemal suspensions prepared from rabbit testes (see above references), detailed, definitive, and more complete investigations can only be carried out with treponemes obtained as a result of either "in vitro" cultivation or large-scale separation techniques. Thus, it is felt that intense efforts should be applied in both these directions.

Since the discovery in 1905, of T. pallidum as the etiologic agent of syphilis, numerous investigators have attempted to culture the organism "in vitro" without success; these investigations have been well summarized in two World Health Organization reports (Wilcox and Guthe, 1966; WHO Scientific Group, 1970). The great majority of investigations relating to growth of the organisms in tissue culture were made on the assumption that treponemal multiplication would be reflected by an increase in the number of actively motile (presumably virulent) organisms in the extracellular tissue culture fluid. As a result, this assumption led to the discard of culture exhibiting non-motile treponemes, usually within one to two days after inoculation.

During collaborative studies, with Dr. John A. Sykes, relating to the ultrastructure of T. pallidum and the pathogenesis of disease in the experimental rabbit and in man, it was demonstrated that (1) T. pallidum takes up an intracellular residence within the parenchymal cells of the infected rabbit testes (Sykes and Miller, 1971); the infected labium (Sykes, Kalan and Miller, 1974); and the human cervix (Sykes and Kalan, 1975); and (2) ultrastructural differences exist between the pathogenic and non-pathogenic treponemes (Sykes and Miller, 1973). The finding of T. pallidum within tissue cells "in vitro" led to the suggestion that an "in vitro" intracellular relationship between mammalian cells and

T. pallidum might be vital to its multiplication in a manner analogous to viral and bacterial replication. Further, the characteristic differences in ultrastructural morphology between T. pallidum (rabbit adapted or human strains) and non-pathogenic treponemes provide a useful marker for preliminary identification of replicating organisms in "in vitro" cultivation systems in which host-indigenous, avirulent treponemes may also become established. Thus, studies were initiated with Dr. John Sykes and Dr. Thomas Fitzgerald (a postdoctoral research fellow) in an effort to explore the possible entry of the rabbit-adapted Nichols strain of T. pallidum into the cells of tissue culture monolayers. Secondary cultures derived from non-infected rabbit testes were established utilizing Eagle's minimum essential medium (MEM) without antibiotics, 20 mH Hepes as a buffering agent, and supplemented with 20% heat-inactivated fetal calf serum. In the initial experiments, 3 ml of a T. pallidum suspension prepared in the tissue culture basal medium supplemented with either 20% heat-inactivated calf serum or with 10-50% heat-inactivated normal rabbit serum and containing 10 to 40 actively motile organisms per high dry darkfield, was added to the monolayer cultures. Incubation was carried out aerobically at 35°C. Examination of the tissue culture fluid showed a decrease in the number of treponemes with time, suggesting that the organisms may have attached to or taken up an intracellular location (Fitzgerald, Miller, and Sykes, 1975). Electron micrographs confirmed the hypothesis and demonstrated that some organisms had either gained entry into or attached to the cells of the monolayer within 30 minutes post-inoculation (Fitzgerald, Miller, and Sykes, 1975). The development of characteristic lesions following the intradermal inoculation of rabbits with cells from washed monolayer cultures at various periods of time after treponeme introduction, provided clear evidence for the presence and persistence of T. pallidum in its virulent form within or attached to cells for at least 96 hours (Fitzgerald, Miller and Sykes, 1975).

Experiments utilizing the Sykes-Moore chamber (Sykes and Moore, 1959) have allowed the actual phase contrast visualization of approximately 95% of inoculated motile T. pallidum attaching to rabbit testis,